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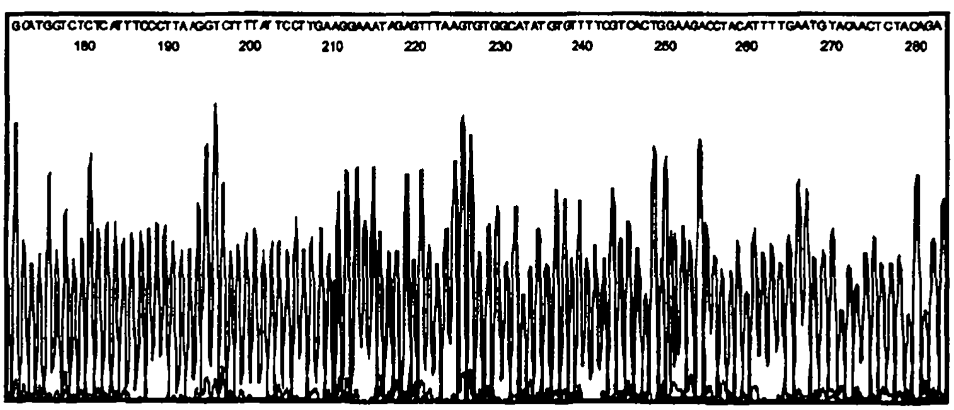

(57) Abstract

Solutions are disclosed which improve the efficiency of DNA sequencing reactions using substituent-labeled, e.g., dye-labeled ddNTPs, and thermostable DNA polymerase. The components of the solution include an effective concentration of manganese ion and a metal ion buffer compound. The use of such solutions is also described.

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DESCRIPTION

A METHOD, REAGENT SOLUTION AND KITS FOR DNA SEQUENCING

Field of the Invention

The present invention relates to buffer solutions for use in DNA sequencing and to the use of such solutions.

Background of the Invention

The following is a discussion of the relevant art, none of which is admitted to be prior art to the appended claims.

DNA sequencing by the Sanger, or chain termination method, has been in common use for some years. The sample to be sequenced may be split into four portions, and each portion may be hybridized to a suitable primer and the primer extended using a DNA polymerase and deoxynucleotides. The incorporation of different dideoxynucleotides in the reaction mixture terminates the chain extension reaction at consecutive positions so that a collection of DNA fragments is obtained each differing by one nucleotide. Frequently T7 DNA polymerase or *E.coli* DNA polymerase is used in such a procedure. Tabor and Richardson (*Proc. Natl. Acad. Sci. USA* 86:4076-4080 (1989)) indicate that the substitution of manganese ions for magnesium ions, which act as a cofactor in DNA sequencing using T7 DNA polymerase or *E. coli* DNA polymerase, reduces the discrimination of these polymerases for dideoxynucleotides (ddNTPs) by 4-100 fold and in *J. Biol. Chem.* 265:8322-8328 (1990) describe the use of manganese ions and pyrophosphatase to generate dideoxy-terminated fragments of uniform intensity using T7 DNA polymerase. However, these enzymes do not operate optimally when dye-labeled ddNTPs are used in the sequencing reaction.

A major improvement in the use of Sanger sequencing has been the discovery of a class of thermostable DNA polymerases which incorporate dideoxynucleotides (ddNTPs) as efficiently as deoxynucleotides, thereby enabling the concentration of the former to be reduced, greatly facilitating the sequencing process (see European Patent No. 655506 B1).

Thermostable Pol I family DNA polymerases in which a phenylalanine in the nucleotide binding domain has been replaced by tyrosine and the exonuclease activity reduced or deleted are particularly advantageous. Such polymerases have been marketed by the ABD division of the Perkin-Elmer Corporation under the trademark AMPLITAQ FS and by Amersham Life Science under the trademark THERMO_SEQUENASE - these are both mutated *Thermus aquaticus* enzymes.

In using Pol I family DNA polymerases containing the tyrosine for phenylalanine substitution and ^{32}P labeled dideoxynucleotides, Tabor & Richardson (*Proc. Natl. Acad. Sci. USA* 92:6339-6343 (1995)) reported that the substitution of manganese for magnesium in the reaction mixture had no significant effect on the use of ddNTPs by *E. coli* DNA polymerase I F762Y and *Taq* DNA polymerase F667Y.

<u>Summary of the Invention</u>

The results obtained from sequencing reactions when using substituent-labeled, e.g., dye-labeled, ddNTPs and thermostable Pol I family DNA polymerases can be improved by adding a particular concentration of manganese and tartaric acid or an equivalent metal ion buffer. The improved results are observed as less variation in band intensity (peak height) than is usually obtained when using the same dye-labeled ddNTPs without manganese ions.

Accordingly, the present invention provides a solution that contains manganese ion at a concentration of between 0.5mM to 3.0mM and a metal ion buffer at a concentration of 5mM to 50mM. The concentration of manganese is preferably between 0.5mM and 1.0mM, or between 0.5mM and 2.0mM, or between 0.5mM and 2.5mM, with about 0.8mM being most preferable in the absence of a metal ion buffer, and about 1.5 mM being most preferable in the presence of a metal ion buffer. Preferably, the manganese concentration in the solution is less than 3.0mM in a polymerization reaction mixture. The concentration of the metal ion buffer is preferably between 5mM and 50mM.

The solution will preferably be pH buffered to a pH between 6 and 8.5, for example about pH 8. The pH is chosen to be compatible with a sequencing reaction when the solution is

combined with al other needed reagents, if any. A "pH buffer" refers to a material which regulates the concentration of hydronium ion, H_3O^+ (or dissociated protons), in solution. A pH buffer regulates the concentration of hydronium ion or dissociated protons in solution by resisting changes in the concentration of the ion in response to dilutions or to additions or subtractions of acids or bases from the solution. Buffers for regulating pH (as distinct from metal ion buffers) which lack primary amino groups, such as tertiary amine buffers are preferred pH buffering agents. Examples include, HEPPS [N-(2-hydroxyethyl)piperazine-N-(3-propanesulfonic acid)], MES [(2-(*N*-morpholino)ethanesulfonic acid)], PIPES [piperazine-*N,N* -bis(2-ethanesulfonic acid))], MOPS [(3-(*N*-morpholino)propanesulfonic acid)], HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)]). The buffer compound is preferably one which does not interfere with with a sequencing reaction in which the solution is to be used. Preferably, the solution is an aqueous solution.

By "substituent-labeled ddNTP" or "substituent-labeled dideoxynucleotide triphosphate" or "substituent-labeled dideoxynucleotide" is meant a 2'-deoxyribonucleotide analog which has a covalently attached detectable group and which lacks a functional 3'-hydroxyl group, so that it terminates chain elongation catalyzed by DNA polymerase. While a radioactive isotope may be present in a substituent-labeled ddNTP as used in this invention, the substituent detectable group provides an additional atom or group to the ddNTP structure, and therefore does not consist of the replacement of an atom in the ddNTP structure with a radioactive isotope, e.g., ^{32}P. Preferably the substituent label is a dye label, more preferably a fluroescent dye label. Dye labels are chemical groups which are detectable spectrophotometrically, preferably be the emission or reflection of light of characteristic wavelengths. Such nucleotides and appropriate dye labels are well known in the art, for example, as described in Lee at al., 1992, *Nucl. Acids Res.* 20:2471-2483 (describing the effects of dyes and dNTPs on the incorporation of dye-terminators in DNA sequencing); Prober et al., U.S. Patent 5,332,666, issued July 26, 1994 (and related patents 5,242,796 and 5,306,618 (describing method and reagents for DNA sequencing using reporter-labeled (e.g., dye-labeled) chain terminators); and Bergot et al., Internat. Patent Application PCT/US90/05565, WO 91/05060 (describing a set of rhodamine dyes and their use in DNA sequencing using Taq DNA Polymerase with magnesium or manganese, or modified T7 DNA Polymerase with

manganese). In the following description, the term "dye-labeled" is generally used, however, it should be recognized that the buffer solution of the present invention can also provide enhancement with ddNTPs labeled with other detectable substituents. Therefore, the various aspects of this invention include the use of additional substituent-labeled ddNTPs.

By "metal ion buffer" is meant a material which regulates the concentration of free metal ion, such as Mn^{2+}, in solution. A buffer regulates the concentration of a species (e.g., a metal ion) in solution by resisting changes in the concentration of the free ion in response to dilutions or to additions or subtractions of that ion from the solution. Such a buffer can, for example, be a dicarboxylic acid, e.g., an alkyldicarboxylic acid such as tartaric acid, where alkyl is a straight or branched chain of 1,2,3,4,5,6,7, or 8 carbon atoms. Other examples of dicarboxylic acids include oxalic acid, malonic acid, succinic acid, maleic acid, glutaric acid, adipic acid, fumaric acid, glutamic acid, aspartic acid, and phthalic acid. Other metal ion buffers include, for example, citric acid, EDTA (ethylenediaminetetraacetic acid), nitrilotriacetic acid, diethenetriaminepentaacetic acid, *N*-hydroxyethyliminodiacetic acid, 2-(N-morpholino)ethanesulfonic acid, dithiothreitol, and *N,N*-bishydroxyethylglycine). A metal ion buffer may be used in the presence of a pH buffer (i.e., a compound which regulates the concentration of free H^+ in solution). The manganese ion will normally come from a salt, for example manganese sulphate ($MnSO_4$), manganese chloride ($MnCl_2$), or manganese acetate.

By "dicarboxylic acid" is meant any lower alkyl, hydroxyalkyl, or aminoalkyl compound containing two carboxylic acid groups, such as oxalic acid, malonic acid, succinic acid, maleic acid, tartaric acid, glutaric acid, adipic acid, fumaric acid, phthalic acid, glutamic acid, or aspartic acid.

In a related aspect, the invention provides a kit for DNA sequencing which includes reagents necessary for DNA sequencing including manganese and a metal ion buffer, preferably at the concentrations specified above or in concentrations readily diluted to those specified above. Suitably, the kit includes a Pol I family DNA polymerase that contains a tyrosine in the nucleotide binding domain of the polymerase at a position analogous to that occupied by phenylalanine in unmutated DNA polymerase (e.g. at position 667 in *Thermus aquaticus*

DNA polymerase I). Preferably the DNA polymerase is a thermostable polymerase, preferably a *Thermus aquaticus* polymerase that has been mutated to replace the phenylalanine at position 667 with tyrosine and in which the exonuclease activity has been substantially removed, e.g. less than 1% of exonuclease activity remains. Suitably, the kit also includes dye-labeled ddNTPs, e.g. ddATP, ddCTP, ddGTP and ddTTPs.

The manganese and metal ion buffer will conveniently, although not necessarily, be stored separately and only mixed when it is desired to carry out the sequencing reaction. Thus, the kit for DNA sequencing will preferably contain, as a first component, a metal ion buffer together with a buffering agent for regulating the pH to between pH 6 and pH 9, preferably about pH 8, deoxynucleotides and dye-labeled ddNTPs, and, as a second component, manganese ion together with a buffering agent for regulating the pH to between pH 3 to pH 7, preferably about pH 6, for example 2-(N-morpholino)ethanesulfonic acid. A DNA polymerase may also be included in the kit, preferably a DNA Pol I polymerase and most preferably a thermostable DNA polymerase, that contains a tyrosine in the dideoxy binding domain at a position analogous to that occupied by phenylalanine in unmutated polymerase. The kit may also contain a pyrophosphatase, for example, an inorganic pyrophosphatase such as that from *Thermoplasma acidophilum*. The polymerase and pyrophosphatase will conveniently be included in the first component of the kit. The kit may also contain sources of other metal ions, for example magnesium and conveniently salts of such metal ions. These additional optional metal ions will conveniently be in the second component of the kit.

The concentration of the manganese ions and metal ion buffer will be such that they can be mixed directly to give the concentrations required for the sequencing reaction or they can be diluted readily to give the required concentrations. While the second component containing the manganese ions is buffered to about pH 6, the mixture resulting from adding the two components together will have a pH of about 8, e.g. pH 7 to pH 9.

In a further aspect, the present invention provides a method for sequencing DNA which comprises performing a DNA sequencing reaction in the presence of dye-labeled ddNTPs and manganese, at a concentration of between 0.5mM and 3mM, and preferably also in the

presence of a metal ion buffer, for example tartaric acid, at a concentration of between 5mM and 50mM, in the reaction mixture.

In a further aspect, the present invention provides a solution containing a metal ion buffer together with a buffering agent for regulating the pH to about pH 8, deoxynucleotides, and dye-labeled ddNTPs. Preferably the solution also contains a thermostable Pol I family DNA polymerase, more preferably a polymerase which has a tyrosine substituted for a phenylalanine in the nucleotide binding site.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures

Figure 1 shows DNA sequencing results from an automated fluorescent DNA sequencing apparatus (ABI model 373 instrument) for an exemplary sequencing in which the polymerization step was carried out in the presence of manganese and a metal ion buffer.

Figure 2 shows DNA sequencing results corresponding to the results shown in Fig. 1, except that manganese was not included in the reaction mix.

Figure 3 shows the structure of the four dye labeled dideoxynucleotides utilized in the examples below. Each of the structures is identified with a Roman numeral, which is referenced in the solution preparation in Example 1.

Description of the Preferred Embodiments

The following examples serve to illustrate formulations of the present invention and their use in a sequencing reaction. These examples are in no way intended to limit the scope of the invention.

Example 1 - Solution Preparation

In the following "pre-mix" protocol all the reagents are contained in two solutions; reagent mix A and reagent mix B.

Reagent Mix A

The following reagents were combined to make 10ml of reagent mix A. (Roman numerals next to each of the ddNTPs designate the attached label as shown in Figure 3.):

2.5 ml 1 M HEPPS N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid) pH 8.0
500 µl 1 M tartaric acid pH 8.0
50,000 units Thermo Sequenase DNA polymerase
2 units *Thermoplasma acidophilum* inorganic pyrophosphatase
100 µl 100 mM dATP
100 µl 100 mM dTTP
100 µl 100 mM dCTP
500 µl 100 mM dITP
9.375 µl 100 µM dye-labeled ddATP (I)
90 µl 100 µM dye-labeled ddCTP (II)
6.75 µl 100 µM dye-labeled ddGTP (III)
165 µl 100 µM dye-labeled ddTTP (IV)
10 µl 50 mM EDTA
1 ml glycerol

The volume was made up to 10 ml with deionized H_2O.

Reagent Mix B

The following reagents were combined to make 10 ml of reagent mix B:

10 µl 1M MES 2-(N-morpholino)ethanesulfonic acid. pH 6.0
200 µl 1M $MgCl_2$
75 µl 1M $MnSO_4$

The volume was made up to 10 ml with deionized H_2O.

Example 2 - DNA Sequencing

4 μl reagent mix A, 4 μl reagent mix B, 200 ng M13mp 18 DNA, 5 pmole of primer (M13 - 40 Forward 5'-GTTTTCCCAGTCACGAC), and deionized water to a total volume of 20 μl were mixed together and subjected to 25 cycles of 96°C 30 seconds, 50°C 15 seconds, and 60°C 4 minutes in a thermal cycler. After cycling, 4μl of a solution which contained 1.5 M sodium acetate, 250 mM EDTA was added. The solution was mixed and 4 volumes (100μl) of ethanol added. The DNA was precipitated by incubation on ice for 15-20 minutes followed by centrifugation. The supernatant was removed and the pellet was washed with 70% ethanol, dried and resuspended in 4μl of formamide containing loading dye. The resuspended DNA was then run on an automated fluorescent DNA sequencing apparatus (ABI model 373 instrument). The result from the machine of the DNA sequence is shown as Figure 1. The corresponding result when the manganese was not included in the reaction mix is shown in Figure 2.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The solutions, kits, methods, and specific compounds described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. For example, those skilled in the art will recognize that the invention may be

practiced using a variety of different metal ion buffers, pH buffers, dye labels, and/or concentrations of manganese, as well as additional reaction or kit components.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Thus, additional embodiments are within the scope of the invention and within the following claims.

Claims

What is claimed is:

1. A solution comprising manganese ion at a concentration of between 0.5mM to 3mM, a metal ion buffer at a concentration of 5mM to 50mM, and substituent-labeled dideoxynucleotides.

2. The solution of claim 1, wherein said substituent-labeled dideoxynucleotides are dye-labeled dideoxynucleotides.

3. The solution of claim 2, buffered to a pH between 6 and 8.5.

4. The solution of claim 2, wherein said manganese ion is at a concentration of between 0.5mM and 2.0mM.

5. The solution of claim 2, further comprising a thermostable DNA Pol I polymerase.

6. The solution of claim 5, wherein said thermostable DNA Pol I polymerase has a tyrosine replacing a phenylalanine in the nucleotide binding domain.

7. A method for sequencing DNA comprising the step of: performing a DNA sequencing reaction in the presence of substituent-labeled dideoxynucleotides, manganese at a concentration of between 0.5mM and 3mM, and a metal ion buffer at a concentration of between 5mM and 50mM.

8. The method of claim 7, wherein said substituent-labeled dideoxynucleotides are dye-labeled dideoxynucleotides.

9. The method of claim 8, wherein said DNA sequencing reaction is performed in the additional presence of a pyrophosphatase.

10. The method of claim 8, wherein said sequencing reaction is performed in the presence of a thermostable DNA Pol I polymerase.

11. The method of claim 10, wherein said thermostable DNA Pol I polymerase has a tyrosine replacing a phenylalanine in the nucleotide binding domain.

12. A kit comprising

a first component comprising a metal ion buffer together with a buffering agent for regulating the pH to about pH 8, and substituent-labeled dideoxynucleotides; and

a second component comprising manganese ion together with a buffering agent for regulating the pH to about pH 6.

13. The kit of claim 12, wherein said substituent-labeled dideoxynucleotides are dye-labeled dideoxynucleotides.

14. The kit of claim 13, further comprising a thermostable DNA Pol I polymerase.

15. The kit of claim 14, wherein said thermostable DNA Pol I polymerase has a tyrosine replacing a phenylalanine in the nucleotide binding domain.

16. The kit of claim 13, further comprising a pyrophosphatase.

17. A solution comprising a metal ion buffer together with a buffering agent for regulating the pH to about pH 8, a first plurality of deoxynucleotides and a second plurality of substituent-labeled dideoxynucleotides.

18. The solution of claim 17, wherein said substituent-labeled dideoxynucleotides are dye-labeled dideoxynucleotides.

19. The solution of claim 18, further comprising a thermostable Pol I family polymerase, wherein a tyrosine is substituted for a phenylalanine in the nucleotide binding site.

20. A method for sequencing DNA which comprises the step of: performing a DNA sequencing reaction in the presence of substituent-labeled dideoxynucleotides and manganese at a concentration of between 0.5mM and 3mM.

21. The method of claim 20, wherein said substituent-labeled dideoxynucleotides are dye-labeled dideoxynucleotides.

22. The method of claim 21, wherein said DNA sequencing reaction is performed in the additional presence of a pyrophosphatase.

23. The method of claim 21, wherein said sequencing reaction is performed in the presence of a thermostable DNA Pol I polymerase.

24. The method of claim 23, wherein said thermostable DNA Pol I polymerase has a tyrosine replacing a phenylalanine in the nucleotide binding domain.

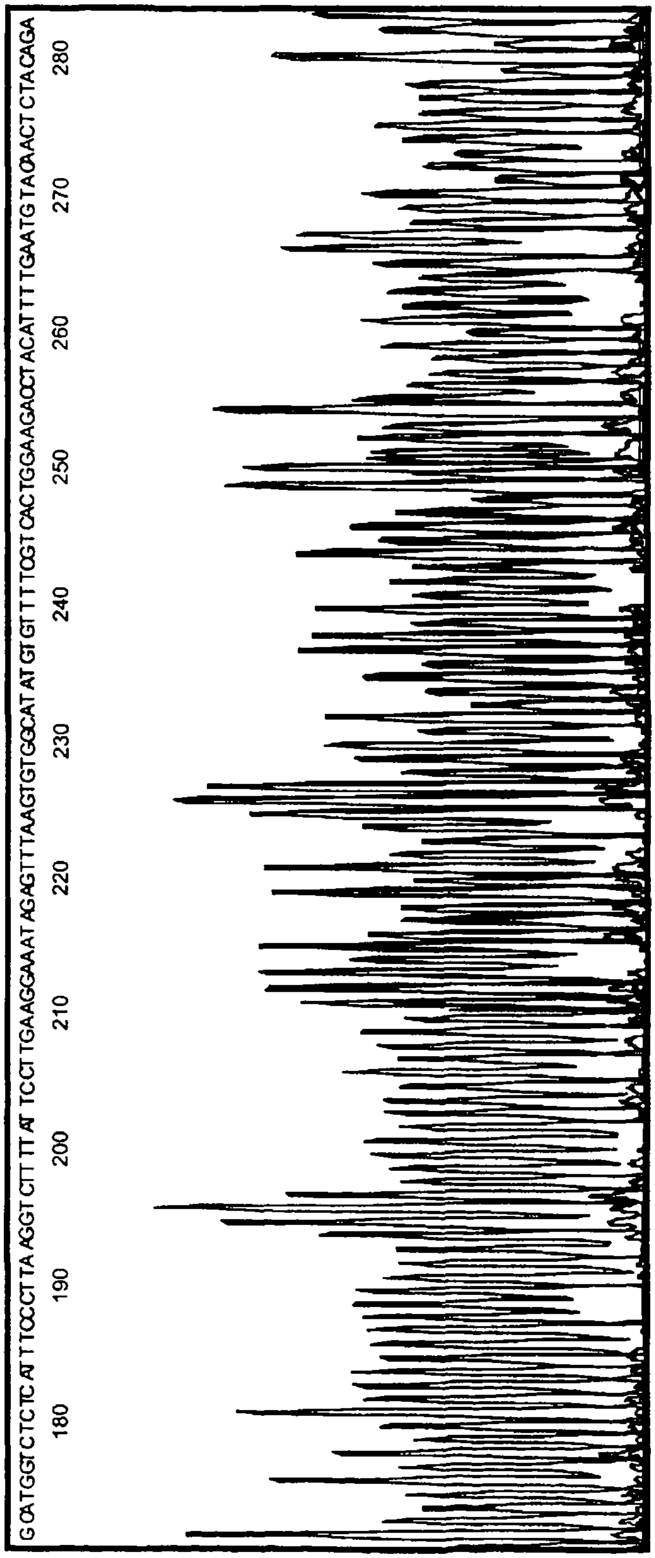


FIG. 1

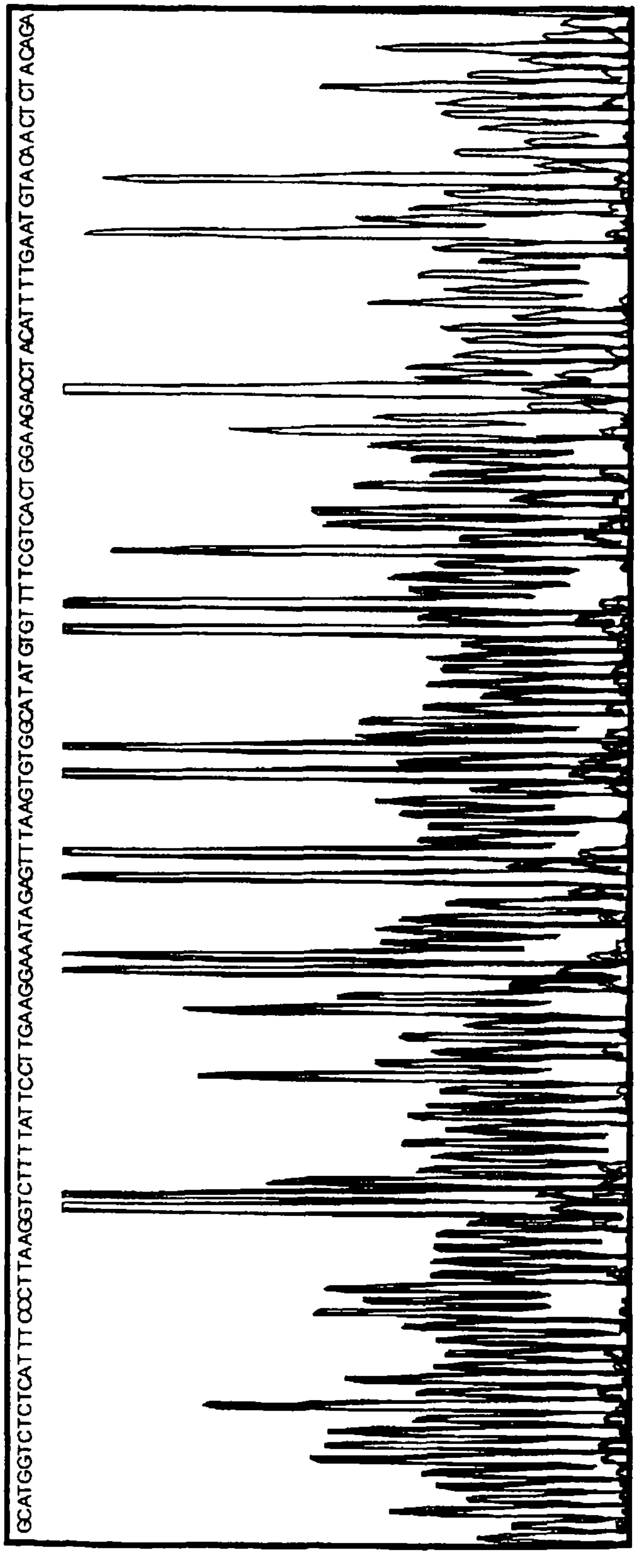
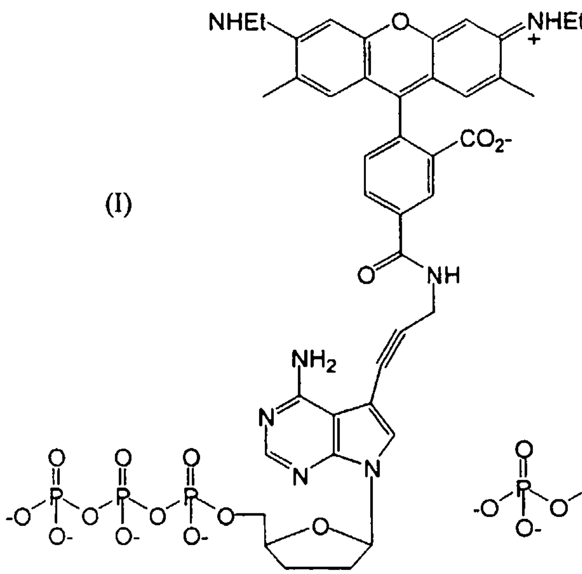

GCATGGTCTCTCAT TT CCCT TAAGGTCTTT TAT TCCT TGAAGGAAATAGAGTT TAAGTGTGGCAT AT GTGT TT TCGTCACT GGAAGAGACT ACAT T TGAAT GTACAACT CT ACAGA

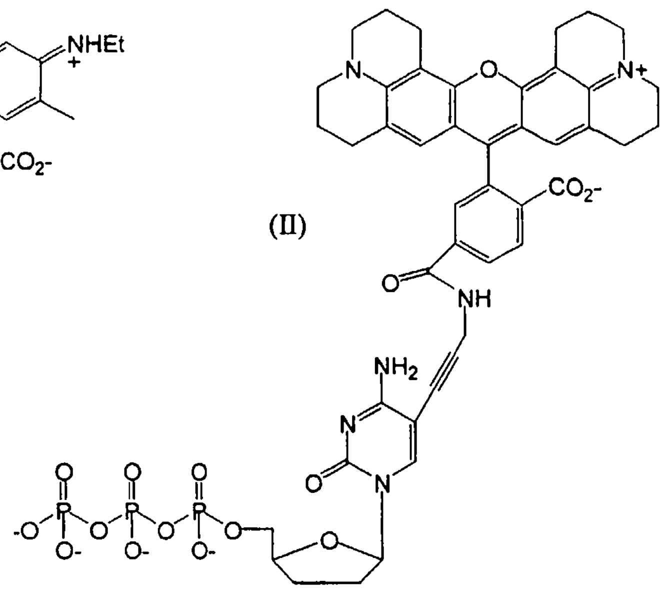
FIG. 2

(I)



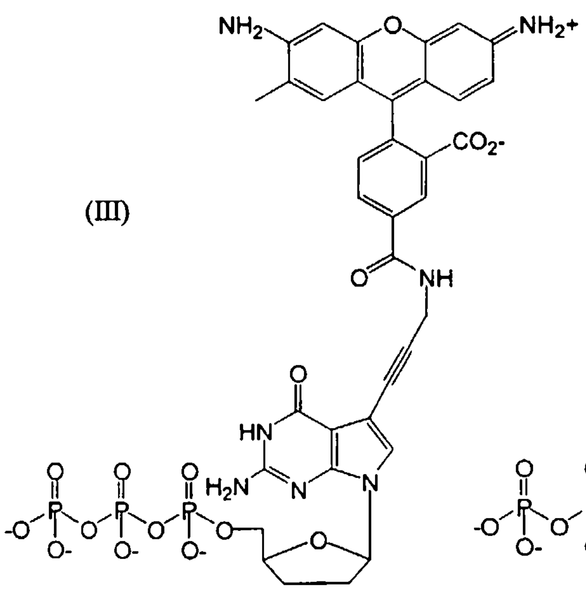
R6G-ddATP

(II)



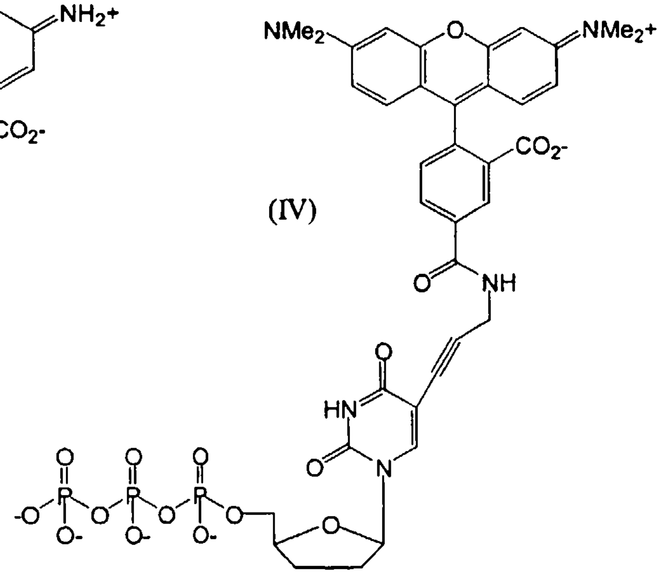
ROX-ddCTP

(III)



R110-ddGTP

(IV)



TMR-ddTTP

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01084

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12P 19/34; C07H 19/04, 19/048
US CL : 435/6, 91.1, 91.2; 536/26.6, 26.7, 26.8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2; 536/26.6, 26.7, 26.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TABOR, S. et al. Effect of manganese ions on the incorporation of dideoxynucletoides by bacteriophage T7 DNA polymerase and Escherichia coli DNA polymerase I. PNAS. June 1989, Vol. 86, pages 4076-4080, see entire document.	1-24
Y	PARKER, L.T. et al. AmpliTaq DNA polymerase, FS Dye-Terminator Sequencing: Analysis of Peak Height Patterns. October 1996, Vol. 21, No. 4, pages 694-699, see entire document.	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 MARCH 1999	16 MAR 1999

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B. FIELDS SEARCHED

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APS, STN WPIDS, BIOSIS,MEDLINE,CANCERLIT,BIOTECHS,LIFESCI,CAPLUS,EMBASE
search terms: manganese, MN, metal ion buffer, sequencing, ddNTPs, label, pyrophosphatase, DNA polymerase I, thermostable, amplitaq, dideoxynucleotides, pH